Defining Immune Cell Profiles and their Role in Predicting Therapeutic Response in Multiple Myeloma

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Abstract

Multiple myeloma (MM) is a plasma cell malignancy that presents with bone marrow plasmacytosis, monoclonal paraproteinemia, and associated end organ damage, including anemia, renal dysfunction and bone disease, amongst others (23, 38, 39). The growth and survival of myeloma cells is driven by inherent genomic perturbations as well as influence of the bone marrow microenvironment (17). Numerous constituents of the bone marrow (BM) microenvironment, including immune cells, are affected both directly and indirectly by MM cells, leading to immune cell dysfunction. The most prominent immune deficiency lies in the humoral immune response, which manifests as low uninvolved immunoglobulin (UIg) levels in serum. We hypothesize that observed low UIg levels and immune dysfunction are due to alterations in the developmental stages of the B cell-compartment in MM bone marrow and associated T and B cell subset alterations; and that B and T cell subset analysis can aid in understanding the immune abnormalities in MM. To address this hypothesis, we utilized multicolor flow cytometry to study the B cell compartment in BM samples from normal donor, smoldering, newly-diagnosed and relapsed MM patients, and to discern immune cell-subsets we utilized the PBMCs collected from newly-diagnosed MM patients enrolled in the DFCI-IFM 10-106 study (2). Patients in this study received state-of-the-art combination therapy with lenalidomide (immunomodulatory agent), bortezomib (proteasome inhibitor) and dexamethasone (steroid) (RVD). We planned to assess how B cell subsets and imbalances in the T cell-

compartment contribute to the framework of the disease, and to distinguish immune parameters that can be useful to predict outcome following RVD treatment. Our analysis of BM samples showed that pro-B cells with renewal capacity were lower in the BM of smoldering myeloma (SMM) (12 ±3.1; $p<0.05$), relapsed/refractory myeloma (RRMM) (3.1±1.6; $p<0.05$) and MM (19.2±4.9) compared to normal BM samples (NBM) (25.1±3) suggesting that aberrations in B cell development may contribute to suppressed UIg in MM. Next, we evaluated PBMCs from patients enrolled on the 10-106 study and achieved complete remission (CR) (N=13) with those not achieving CR (N=18) following therapy. We observed significant differences in patients achieving CR versus those not achieving CR in B2 cells (63.6±4.6l vs. 42.1±3.9, respectively; $p<0.05$), CD3 cells (61±2.7 vs. 38.9±5.7, respectively; $p<0.05$); CD4 cells (63.4±3.7 vs. 37.1±5.5, respectively; $p<0.05$); Th2 cells (73±6.1 vs. 47.7±5.9, respectively; $p<0.05$), and PD-1 expressing CD8 T cells (63.2±8 vs. 39.5±6.1, respectively; $p<0.05$) and decrease in Th1 cells (23.3±5.6 vs. 46.3±5.4, respectively). These differences were also observed when absolute numbers of each cell type were computed. Our preliminary results suggest the potential of these markers measured at diagnosis to predict CR in MM patients following RVD treatment. This data warrants a larger study in this patient population, to decipher how B cell function is affected in MM, and what subsequent effects occur on cell-mediated immunity controlled by T helper cells.

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Chapter One: Introduction

1.1 Background

Overview: Multiple Myeloma

Multiple myeloma (MM) comprises thirteen percent (38, 32) of all hematologic cancers, and is acknowledged as the neoplastic cancer of plasma cells in the bone marrow microenvironment. Conventionally, plasma cells arise in the bone marrow after B cell development in the lymphocyte lineage of hematopoiesis, and are active in fighting infections through antibody production (3). Short-lived plasma cells propel an immediate immune response from the red pulp of the spleen, whereas long-lived plasma cells inhabit the bone marrow, producing 80% of specialized serum antibody over time (36). The defining characteristic of myeloma is the post-germinal center, clonal proliferation of abnormal plasma cells and their production of monoclonal antibody protein (M protein) (**Figure 1**). Accumulation of M protein in the serum of patients can lead to eventual kidney damage, and is often used as a clinical tool to measure disease progression (24).

Before the official diagnosis of myeloma, a premalignant, asymptomatic stage known as a monoclonal gammopathy of undetermined clinical significance (MGUS) occurs in the bone marrow (15, 38, 47). Here, serum monoclonal M protein levels are below 3g/dL, and less than 20% of clonal plasma cells are present (24). Smoldering myeloma (SMM) is the next stage increasingly prone to malignancy, where measurements of the former two criteria have escalated, however, this stage remains primarily asymptomatic (47). Both of these stages can progress into symptomatic, malignant, newly diagnosed myeloma (MM) upon observance of 10% clonal

plasma cell content in the bone marrow, and significant monoclonal (M) protein observance in the urine or serum (24). Progressive MM accounts for the major part of the disease course, featuring an afflicted immunoglobulin isotype, the most common being IgG, followed by IgA, IgD, IgM, and IgE myeloma (34). Myeloma's cyclic prevalence is due to extremely high relapse rates, which are considered an integral part of the disease course, as a majority of patients do relapse (22, 28). This leads to the necessity of classifying a relapse/refractory (RRMM) stage, consisting of patients failing to respond to viable, ongoing, advanced treatment protocols (28).

A prominent signature of this B cell malignancy is persistent immune deficiency, which predisposes patients to infectious complications by suppressing development of anti-myeloma immune responses, leading to poor prognosis. Patient demographics reveal a median diagnosis at the age of 70, and minimal survival rates (less than 30%) for those less than 60 years of age (28, 38). Effective therapy for myeloma has been high-dose chemotherapy and with bone-marrow transplantation, which limits the elderly patients' eligibility for treatment, primarily due to their age and performance status in combination with the state of the disease (28). Novel treatment protocols introduced in recent times, which have notably improved survival, involve the immunomodulatory drugs: Lenalidomide (Revlimid), Pomalidomide (Pomalyst), Boretzomib (Velcade) (2, 28, 38). Within current therapeutic strategies, Dexamethasone is a corticosteroid utilized with the proteasome inhibitory drug Velcade, and immunomodulatory drug Revlimid (RVD) (2). The combination of these drugs targets most stages of MM, by manipulating protein homeostasis, inhibiting tumor angiogenesis, terminating proliferation of tumor cells, and inducing apoptosis of malignant cells (31). These emerging treatment strategies provide an advantage to patients by manipulating the immune system's ability to eliminate malignant

plasma cell clones, and eliminating the risk of 'graft-*versus*-myeloma' due to minimally successful allogeneic stem cell transplantation (22).

Consistent monitoring of myeloma progression involves clinical terminology that aids in categorizing the degree of therapeutic progress after treatment. Recently, labeling the phenotypic presence of myeloma in the bone marrow has become more effective with the introduction of minimal residual disease (MRD) classification. MRD is a highly sensitive analysis that can detect 1 malignant plasma cell in 10,000 bone marrow cells (2), and is used to determine subclinical levels of myeloma (37). MRD negative patients have a better prognosis than do MRD positive patients, predicting a lower likelihood of relapse, and a higher chance of progression free survival (PFS) (37). Complete response (CR) is a vital standard to measure the response to treatment by having bone marrow plasma cell levels fewer than 5% without presence of clonal plasma cells (16) as well as lack of M protein in the serum (2). Other therapeutic response criteria for prediction of PFS, which fall under the opposite spectrum category: "non CR," are partial response (PR), very good partial response (VGPR), and near CR (nCR) (16). Because such prognostic criteria influences relapse rates, it is beneficial to correlate MRD and CR diagnoses to determine a viable predictive clinical outcome.

10-106 DFCI-IFM Study

Revlimid Velcade Dexamethasone (RVD) combination therapy has yielded favorable PFS in current treatment protocols for myeloma. A collaborative initiative to study RVD by Dana Farber Cancer Institute (DFCI) and the Intergroupe Francophone du Myélome (IFM), led to the analysis of 700 IFM and 690 DFCI myeloma patient samples, to evaluate response to therapy and overall as well as progression free survival (PFS). The study, applied to all samples,

incorporates two phases, an induction phase followed by a consolidation phase, where two groups of newly diagnosed myeloma patients received RVD treatment cycles with or without autologous stem cell transplantation (2) (**Figure 2**). The groups were stratified by age (<65 years), and exhibited measurable, symptomatic, mostly IgG myeloma, samples were collected before induction therapy. Between both groups in the IFM 700 sample analysis, PFS was improved in the group with RVD plus transplantation, though overall survival curves were similar (2). Furthermore, response rates (no visible MRD/full CR) were higher in the transplantation+RVD group, around 79%/59%, than in the RVD-alone group, 65%/48% (2). Patients had responses to treatment (CR determination) at any time point in the treatment plan in Figure 2. These results warrant the analysis of probable trends between CR and non-CR patient groups, regardless of treatment administered.

Our notion is to compare patient samples in the USA component of the study to observe significant differences in the in immune cell profiles of myeloma patients who have undergone this RVD treatment protocol. Though distinguishing the group who received transplantation+RVD from the RVD-alone group is currently unavailable until the completion of the USA patient study, patient CR profiles were confirmed and reported before maintenance therapy (2). By approaching patient categories achieving CR or Non-CR (nCR, VGPR, PR) in response to RVD, principal immune variables that suggest key divergences, can be classified. With this approach, there is hope that crucial B and T cell signatures distinguishing patients at diagnosis can be effectively translated as a baseline for predicting CR. Our primary goal does not center on developing effective therapeutic outcomes, understanding toxicities, or finding correlation with progression-free or overall survival, rather, it centers on validation of such a potential to be able to predict complete response (CR) retrospectively, for the proposed

therapeutic interventions at the time of diagnosis or start of the treatment regimen, on the basis of one or more immune parameters in myeloma.

Immune Cell Exploration

Although anti-MM immune responses have been reported following vaccination, correlative clinical responses are not observed (24). This inadequacy is partially explained by significant immune dysfunction. Over the years, the depth of data regarding T cell dysfunction in MM has been substantial. Here, dysfunction has been applied to Th1, Tregs and Th17 cells (42, 43), iNKT cells, plasmacytoid dendritic cells (pDC's), myeloid-derived suppressor cells (MDSC's), and peptide-based immune responses in myeloma (3, 9, 10, 11, 20). For instance, increased production of IL-17 by elevated levels of Th17 cells promotes myeloma growth and survival (43), and high Th17 levels affect immune function of other T cell types, such as Th1 cells. In addition, FOXP3+ regulatory T cells are dysfunctional in myeloma, which may be cause of an imbalance in immune homeostasis (42). This potentiates that other immune compartments may contain immune populations that are out of proportion, which is crucial to uncover in progressive myeloma (MM). The depth of analysis of T cell behavior in MM compels the question of how humoral immunity plays into this picture, which surprisingly, has rarely been explored in MM. However, there is anticipation and suggestive evidence in the literature generalizing that B-T cell-cognitive cooperation is critical for the development of robust adoptive immune responses against infectious diseases and cancer cells (35). In addition, uninvolved immunoglobulin suppression (UIgs) is prominently noted (i.e. suppression of serum IgA and IgM in IgG myeloma) (19). All in all, very limited information is available regarding B cell function and cause for this UIg suppression. Since the acquisition of antibody repertoire occurs outside of the BM (i.e. in the germinal center of the lymph node/spleen), and is a critical

step in follicular B cell-maturation that hinges on B cell-development in the BM, it is necessary to explore a potential link between the production of normal threshold levels of uninvolved immunoglobulins in relation to plasma cell malignancy.

B Cell Development

Originating in the bone marrow, B cell development begins from a hematopoietic stem cell's (HSC) commitment to a common lymphoid progenitor cell (CLP), followed by events that convey succession to the B cell lineage (40). Subsequently, an early B cell goes through four distinct phases, Pro-B, Pre-B, Immature, and Transitional. In the Pro-B phase, the premature B cell still maintains a stem cell compartment, accounting for its plasticity and renewal capabilities. As it becomes a Pre-B cell, the B cell receptor (BCR) starts to fully form, and the cell is fully committed to become a B cell (7, 21). After the BCR matures, the cell is recognized as an immature B cell and is released into the periphery. The cell circulates as a transitional cell (T1/T2/T3) to the lymph nodes and spleen, where participation in the germinal center reaction and selection produces mature B cells as well as plasma cells (5). In the germinal center, transitional B cells interact with follicular dendritic cells (FDC) and T follicular helper cells (Tfh). In order to evolve into specialized cells, the BCR's of transitional cells undergo somatic hypermutation (SHM) and antibody class-switch recombination (CSR) for antigen specificity (to form specific Ig's) (40). They are then presented auto antigens, and eliminated through negative selection (40). A significant age effect of hematopoiesis in the bone marrow necessitates critical exploration, as myeloma causes substantial bone damage in patients (23). This clinical observation ties in with the fact that myeloma cells sitting in the bone marrow could also have potentially adverse consequences on B cell development. Hence, analyzing precursor B cell stages is essential, as oncogenic events are initiated in the germinal center, since SHM and CSR

are prone to erratic mutations, and late oncogenesis occurs in the bone marrow in MM (6, 12, 14).

B cells are an important component of the immune system responsible for generation of antibody responses against various infectious agents (26). Immature B cells migrate from bone-marrow (BM) to blood to lymphoid organs, particularly spleen, where with the help of BAFF (27) modulation they generate six different B cell-subsets (B1a, IRA-B, B1b, B2, MZB and Bregs) before entry into periphery (1, 11, 27, 48). B1a and B1b are antibody producing cells that are prevalent in early stages of life, B2 cells comprise the majority of conventional B cells, and Bregs help maintain tolerance (1, 11) (**Figure 3B**). From an immune functionality standpoint, B1 cells provide natural antibodies, predominantly IgM, and have a limited capacity, with no memory compartment (1). Below the umbrella of B1 cells, the B1a subgroup is generated in the peritoneal cavity, assists macrophages, and includes Ira-B (1, 11). B1b cells elicit protection against polysaccharide antigens. Recent data has introduced that increased Th17 cells induce raised levels of B1a cells (51) a crucial component in observed atypical TH17 behavior in MM (43). On the other hand, B2 cells primarily have a diverse repertoire and memory compartment (memory B), as they have progressed through the germinal center in the lymph nodes and spleen, and therefore, produce all UIgs (IgG, IgM, IgA, IgE, IgD) (1, 34). B2 cells consist of follicular B (FB) cells in the germinal center, as well as marginal zone (MZ) B cells, which are mostly stagnant in the marginal zone of the spleen, though recent publications imply circulatory behavior in cancer and infections (1, 26, 41). Lastly, Bregs produce IL-10 to maintain immune homeostasis (48). Analysis of these subpopulations in MM can aid in distinguishing how B cell composition and immune function is affected in MM patients.

As cell fate decisions are being made, a precursory hematopoietic stem cell (HSC) commits to the B cell lineage after systematic adaptation of lineage markers. The primary, simplistic flow diagram encompasses the progression of a B cell from its early stages, branching to more specialized stages, and finally major subsets (**Figure 3A**). In order to identify noteworthy differences in B cell subpopulations between MM stages, it is essential to lineage trace myeloma cells using early developmental markers. Distinguishing the markers primarily aids in gating on specific cell populations using flow cytometry: CD34 as a hematopoietic stem cell marker, CD38 as an early B cell marker indicating activation and renewal capacity, CD10 prominence after the E-B stage, and IgM presence on the cell surface as an immature cell. The advantage of this adult bone marrow study delineates stage appropriate markers for their universal application to B cells under myeloma conditions (21) (**Figure 4**). Our aim is to decipher how malignant plasma cells sitting in the bone marrow microenvironment affect B cell development at pro-B, pre-B, and immature B cell points at each myeloma disease stage. To address the basis for the observed decrease in antibody levels, we have chosen to determine the expression profiles of various B cell developmental stages by surface markers in the bone-marrow samples collected from myeloma patients, with multi-color flow cytometry (BD, LSR Fortessa). It is expected that in the progressive stages of myeloma, including MGUS, smoldering, progressive myeloma, and relapse/refractory, there are apparent low uninvolved immunoglobulin levels due to an abnormality in B cell-subset composition in the developmental stages. Within the scope of B cells, and in relation to the 10-106 study, we wish to decipher how subsets (namely B1a, B1b, B2, Bregs, Ira-B, MZB) formed after germinal center maturation, behave under myeloma conditions, in patients who have achieved CR compared to those who have not achieved CR.

Because the picture of immune cell function and how UIgs are suppressed in myeloma is unclear, we propose to zoom into the framework of various immune cell types, in order to identify how the behavior of these populations may play into defining low functional antibody levels. Since low UIgs are key in the prediction of relapse rates, this hypothesis ideally aims to establish further associations (19, 30). Therefore, we predict that myeloma stages will present differences in their B cell developmental compartments in MM bone marrow, and that conventional B and T cell compartments (including B cell subsets, T subsets, T regulatory cells, T cell activation, and T cell profiles measuring cytokines defined in **Table 1**) will be significantly altered in MM patients from the 10-106 study. With this perspective, we hypothesize that low UIg levels alongside observed immune dysfunction are due to differences in the stages of B cell development in the bone marrow of MM patients compared to normal bone marrow, and that analysis of B and T cell subsets in the peripheral blood of myeloma patients in the 10-106 study will bring forth immune parameters that can aid in prospective prediction of CR in MM patients. To address this hypothesis, we utilized multicolor flow cytometry to study the B cell compartment in BM samples from normal donor, smoldering, newly-diagnosed and relapsed MM patients; and to observe immune cell-subsets, we utilized the peripheral blood mononuclear cells (PBMCs) collected from newly-diagnosed MM patients enrolled in the DFCI-IFM 10-106 study. This predictive potential features unveiling of a larger scope of insight to further comprehend imminent relapse in most myeloma patients. Examining lymphocyte development at precursory time points, as well as B and T cell profiles in detail, will help uncover novel information, which can aid in pinpointing success in achieving CR following RVD treatment regimens. In addition, phenotypic profiles of immune populations, which are

perceived in humoral immunity in MM, can be specified to unveil the properties of myeloma at a molecular level.

1.2 Schematic Figures

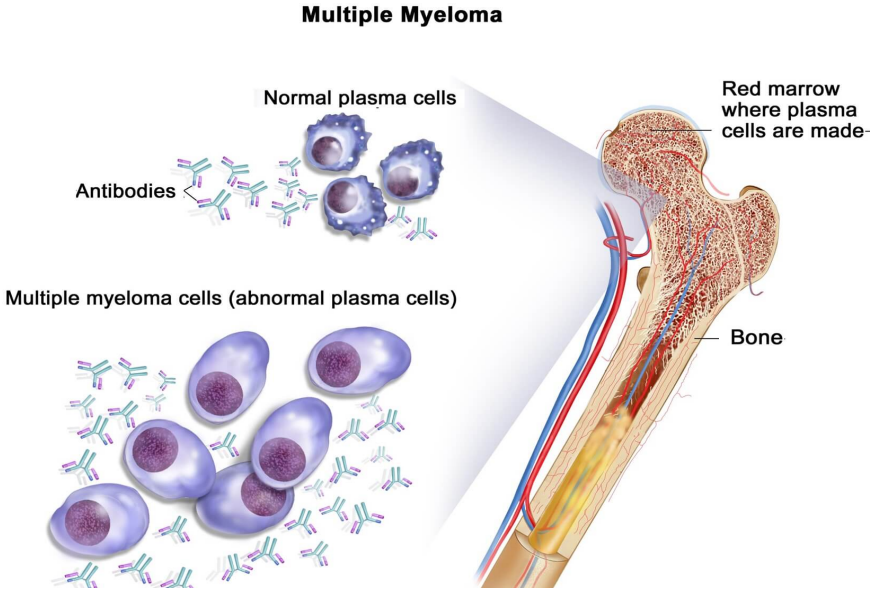


Figure 1. Schematic representation of plasma cell-phenotypic changes in multiple myeloma and abundant presence of M protein. (39)

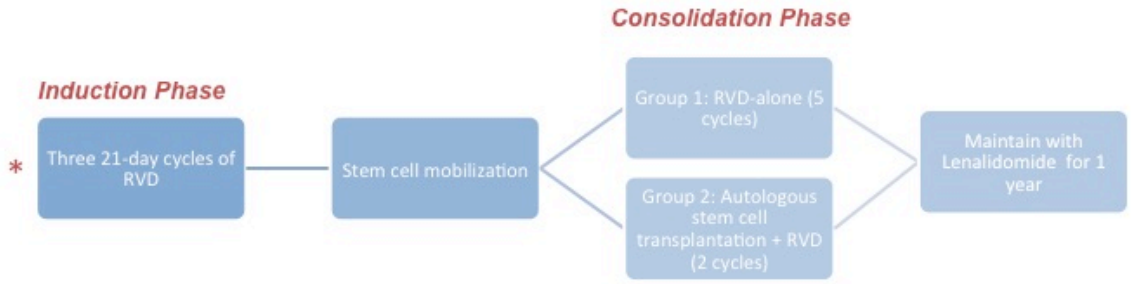


Figure 2: Chronographic treatment protocol with RVD for the DFCI-IFM 10-106 study with 700 myeloma patients. (2) Results are from the French study; 690 patients in the USA were recruited for the study. (*Represents the time point at which diagnostic samples were obtained and frozen.)

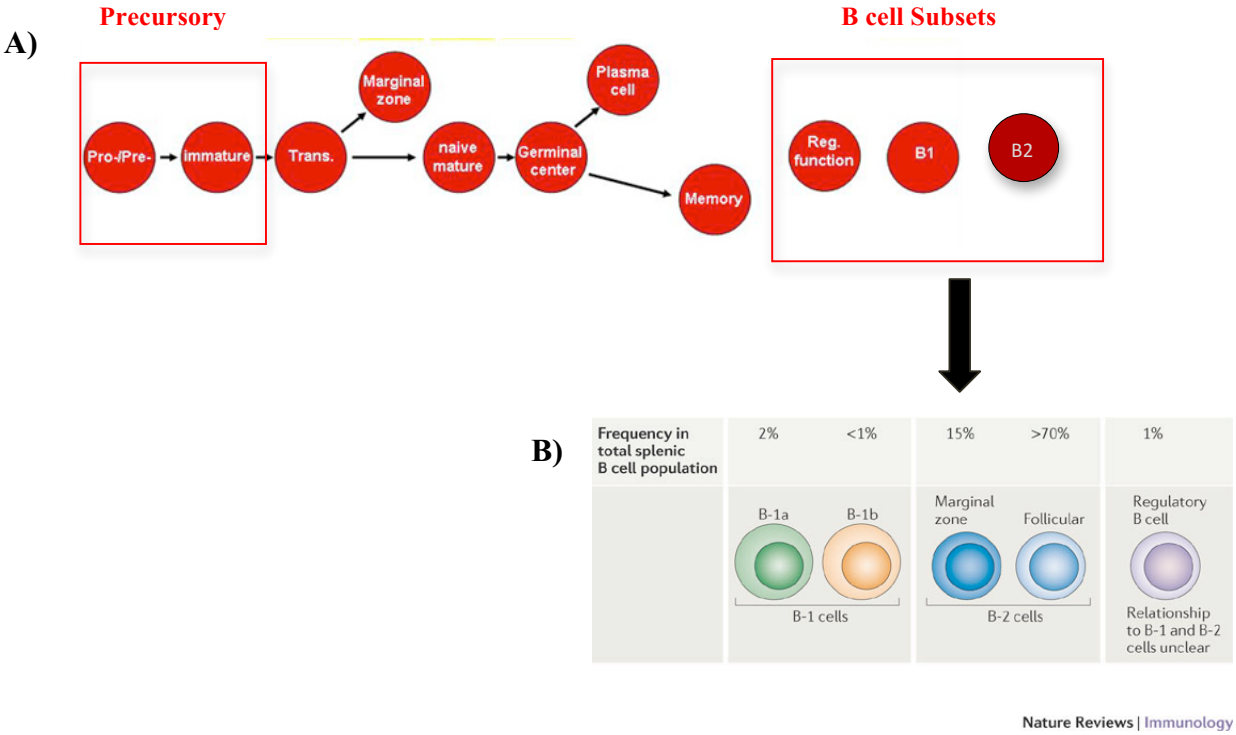


Figure 3: Schematic Overview of B Cell Developmental Stages and B Cell Subsets. (A) Early stages of B cell development are outlined, followed by B cell evolution into various cell types. (29) (B) Further defining B lineage subsets (B1a, B2, Bregs) (4).

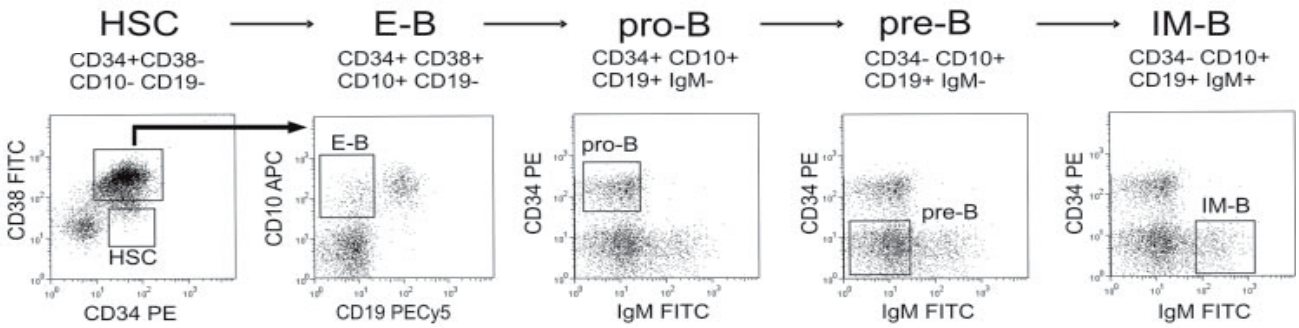


Figure 4: Flow Cytometry Gating Strategy for B cell developmental markers. Figure obtained from *Hystad et. al.* characterization of B cell development in adult bone marrow (21).

Chapter Two: Data

2.1 Analytic Overview

In spite of advanced combinational chemotherapeutic regimens, most multiple myeloma patients will eventually relapse following treatment, regardless of their CR status. In order to address the role of the well-documented immune dysfunction in various immune compartments in the progression of myeloma, as well as response to treatment and eventual relapse, our chief objective in this study is three-fold. Firstly, our analysis of novel differences in B cell lineage developmental compartments is presented by comparing freshly collected normal bone marrow (NBM) with the three different stages (SMM, MM, and RRMM) of the multiple myeloma bone-marrow microenvironment. Secondly, we provide data on B cell subset frequencies and absolute numbers in frozen myeloma patient peripheral blood samples, which were collected at diagnosis. Here, in a retrospective fashion, we show how noteworthy immune parameter(s) are useful in the capability to predict CR or non-CR following current treatment (RVD) in myeloma patients. Finally, our assessment of T cell subsets has offered results on CD4 and CD8 cells, T cell activation, cytokines, and T regulatory cells, in order to understand the scope of their behavioral profiles under RVD treatment conditions. Our samples sets suggested investigation of such significant immune cell populations, in order to decipher patterns that will benefit the characterization of the MM tumor microenvironment, as well as therapeutic response prediction after RVD treatments.

2.2 Materials and Methods

Patient Samples

Both peripheral blood and BM samples were collected from newly diagnosed myeloma patients (MM), Smoldering myeloma patients (SMM), and relapse/refractory patients (RRMM) at the Dana-Farber Cancer Institute. These samples were collected after informed consent in accordance with the Declaration of Helsinki and approved by the institutional review board of the Dana-Farber Cancer Institute. Healthy donor samples were obtained from commercial sources (AllCells, Alameda, CA).

Cell Isolation

For the isolation of peripheral blood mononuclear cells (PBMCs) from peripheral blood, and bone marrow mononuclear cells (BMMCs) from bone-marrow (BM) aspirates, samples were processed with Ficoll® Paque Plus (GE Healthcare) by layering, centrifuging, and washing. Remaining red cells were lysed with Lysis Buffer (BD Biosciences, CA) if required. Cells were resuspended in RPMI 1640 complete media containing antibiotics (penicillin, streptomycin, and amphotericin B, Fisher Scientific, NJ) and 10% fetal bovine serum until further analysis, or frozen with 20% DMSO and kept at -80^{0}C until used. Frozen PBMC were quickly thawed using warm complete RPMI 1640 media and washed twice prior to use.

Cell Surface/Intracellular Staining

The six subsets of B cells mentioned above, T cell-subsets including naïve, central, effector and exhausted memory cells in both CD4 and CD8 compartments, activated T cells with expression of CD40, OX40, ICOS, 4-1BB and PD-1 in both CD4 and CD8 compartments, Tfh cells with expression of CXCR5, regulatory, and Th1/17 subsets in NK, NKT, CD4, and CD8 compartments, Tregs classified as $CD127^{low}$ and $CD25^{high}$ with $FOXP3^{+}$ in CD4 cell compartments, and cytokines were analyzed as follows. A mix of antibodies (**Table 1**) was added to each tube containing 100ul of PBMC, and incubated for 20 minutes at room temperature, after which cells were washed with PBS. T cell, T activation, and Tfh tubes were fixed with fixation buffer (BD Biosciences), and fixation/permeabilization solution (BD biosciences) was added to B cell and Treg tubes. For cytokine stimulation, PBMCs were activated with Leukocyte Adhesion cocktail containing golgiplug (BD biosciences) for 6 hours.

After washing, cells were stained for surface makers and fixed with fixation/permeabilization solution (BD biosciences) overnight. Cells were then washed with permeabilization buffer (BD Biosciences) for 12 minutes at 2000 rpms prior to intra-cellular staining. Consecutively, for B cell, Treg, and cytokine tubes, appropriate monoclonal conjugated antibodies were added: GM-CSF for B cells to identify IRA-B cells within the B-1a subset, Foxp3 for Tregs, and IFN-γ for Th1, IL-4 for Th2, IL-9 for Th9, and IL-17A for Th17, for cytokine tubes. All cell tubes were washed with PBS after half-hour incubation at room temperature and resuspended in PBS prior to analysis at the BD LSR Fortessa.

For B cell developmental cell staining, precursor B cell antibodies, including CD34, CD38, CD10, CD19, IgM, and IgD were used to stain with 100ul of PBMC and incubated for 20

minutes at room temperature, after which cells were washed with PBS and fixed (BD Biosciences) prior to BD LSR Fortessa analysis.

A list of the conjugated monoclonal antibodies that were purchased from BD Biosciences can be found in **Table 1,** for stimulation of each of the immune cells in the tubes.

Flow Cytometry Analysis

Flow analysis was performed using BD LSR Fortessa flow cytometer, and FACS-DIVA software. The 6-tube template worksheet was set up to acquire and analyze stained PBMCs. Each cell-tube was set up with appropriate isotype controls for effective gating, and single-color stained cells with each of the specific antibodies were used to correct the compensation. The BD LSR Fortessa flow cytometer was calibrated daily with CST and calibration beads, prior to analysis of samples, in order to maintain similar results.

Gating was directed on CD38, CD34, CD10, and CD19 for B cell developmental profiles. (**Figure 4**; *Hystad et. al*. (21)).

Initial gates were set up on lymphocytes and further gated on CD19 and CD3 to analyze subsets within B and T populations, respectively. From the CD19 population, gates on CD43 and CD5 were used to distinguish B cell subsets: B1a, B1b, B2, Bregs, memory B cells. The B1a subset was further delineated to GM-CSF, (44) which is characterized as Ira-B, and the B2 subset consisted of FB and MZB cells. From CD3 populations, CCR7 (8) and CD45RA (45) were gated on, to separate naïve, central memory, effector memory and exhausted memory T cell subsets

within CD4 and CD8 populations. Tregs were gated on using CD127 and CD25, as well as their master regulator, Foxp3.

Absolute Cell Number Calculation

Complete blood cell counts (CBC) were obtained with the help of clinical colleagues, from patient data files. We used absolute lymphocyte counts from CBC to calculate total T cells, B cells NK cells, and subset-populations were further obtained from total lineage cell numbers. Absolute cell numbers were only available for non-CR (N=9) and CR patients (N=12), out of total number of patients evaluated for percentages in non-CR (N=18) and CR patients (N=13), respectively, for Figures 5-11. Absolute numbers are expressed per ml.

Statistical Analysis

The mean and standard error of the mean were calculated for all data sets. Statistical significance was ascertained by performing the appropriate Student's T-test, which is listed in the legends of each figure. Significant differences were indicated as $p<0.05$ (marked with an *).

2.3 Results

Profiling of B cell developmental stages ascertains differences between MM BM versus Normal BM (NBM).

After gating on the markers defined by *Hystad et al.* (21), normal bone marrow mononuclear cells (BMMC) were compared to BMMCs from smoldering, newly diagnosed, and relapse/refractory MM (SMM, MM, RRMM). Primary differences included significant depletion of pre-B cells, pro-B cells, and increased immature B cells in the myeloma stages (**Figure 5A**). In the early B cells (having renewal capacity), a significantly high percentage of $CD34^{+}CD38^{+}CD10^{+}CD19^{+}$ precursory cells were seen in RRMM (6.8±2.1) ($p<0.05$), compared with NBM (1.1±0.2) and an increasing trend was seen in SMM (1.2±0.4) and MM (1.9±0.6). Major differences that proved to be of key interest were lower percentages of pro-B cells with renewal capacity in SMM (12 ±3.1; $p<0.05$), RRMM (3.1±1.6; $p<0.05$) and MM (19.2±4.9) compared to normal BM (25.1±3). Significant differences in Pre-B cells among three MM stages and NBM were not observed. In addition, Immature-B cells were significantly higher in RRMM (38.6±7.2) ($p<0.05$) compared to NBM (17.2±1), and increasing trend was seen in SMM (31.5±8). This analysis indicates that pro-B cells with renewal capacity were significantly lower in MM stages compared to NBM, which may have a role in the reduced number of mature B cells and lower levels of UIgs observed in MM (**Figure 5B**).

B cell Subset quantification at diagnosis in patients achieving CR following RVD treatment compared with patients not achieving CR.

Flow cytometry analysis of B cell subsets was initiated by lymphocyte gates using side scatter (SSC) and forward scatter (FSC) to obtain $CD19^+$ cells (B cell marker) from PBMCs, followed by gates on CD43, a B cell malignancy marker (25) and CD5, a B1a cell marker (50) (**Figure 6A**). Gating on these subsets isolated populations B1a ($CD19^+CD43^+CD5^+$), B1b ($CD19^+CD43^+CD5^-$), B2 ($CD19^+CD43^-CD5^-$), and Bregs ($CD19^+CD43^-CD5^+$) as represented in **Figure 6A.** After quantification, important differences in samples at diagnosis were observed between patients achieving CR (N=13) and patients not achieving CR (N=18). B2 cells were significantly elevated at diagnosis in patients achieving CR compared to patients not achieving CR (63.6±4.6 vs. 42.1±3.9; ($p<0.05$). Bregs (19.8±2.6), Ira-B (8±2.2), MZB (10.8±2.7), and FB (26±5.6) cells were significantly lower at diagnosis in patients achieving CR compared to patients not achieving CR (30.4±2.6, 32.8±6, 29.6±5, and 44.3±5.7, respectively, $p<0.05$). (**Figure 6B**).

To further confirm the results, we calculated absolute cell numbers for each cell type using total lymphocyte count. Similar trends were observed for all cell types, considering absolute cell numbers, which adds significance to the observed differences. (**Figure 6C**). Absolute cell numbers were significantly higher at diagnosis in patients achieving CR for B2 cells, showing a strong correlation for validity. Differences observed with absolute cell counts also indicated significant differences between patients achieving CR versus those not achieving CR for B1b and memory B cells, but not for Bregs, IRA-B, MZ and FB cells, which were observed when proportion of cells were considered. (**Figure 6C**).

Comparative frequencies of T, B, NK and CD4 cells at diagnosis in patients achieving CR compared with patients not achieving CR.

Identification of CD4, CD8, and NKT populations, in the PBMCs of myeloma patients at diagnosis in the 10-106 study, was determined by flow cytometry analysis (**Figure 7A**). Within these cell populations, significant differences were observed in T (61±2.7) CD4 (63.4±3.7); and B cells (12.3±3), in patients achieving CR versus those not achieving CR (38.9±5.7, 37.1±5.5, 5.2±1.6, respectively $p<0.05$). (**Figure 7B**). The two NK cell populations, NKT (9.8±2.2) and NK (26.7±2.7) ($p<0.05$) were significantly lower at diagnosis in patients achieving CR compared to those not achieving CR (29.6±4.6, 55.7±6.5, respectively) (**Figure 7B**). Discerning absolute cell counts, T cells showed higher numbers at diagnosis in patients achieving CR compared to those not achieving CR, as seen in **Figure 7B**. However, taking into account absolute cell counts made the CD4, B, and NK cell compartments insignificant between patients who achieved CR vs. those who did not achieve CR, though the trends were similar. CD8 cells were significant when looking at absolute numbers at diagnosis in patients achieving CR compared with patients not achieving CR (**Figure 7C).**

Quantification of circulating memory T cell-subsets in CD4 and CD8 populations at diagnosis in patients achieving CR compared with patients not achieving CR following RVD treatment in MM.

T cell subsets were quantified in PBMCs with respect to the relative proportion of $CD4^+$ and $CD8^+$ T cell (gated on $CD3^+$) populations, and the presence of naïve memory, effector memory, central memory, and exhausted memory cells were determined by gating for CD45RA, a T cell

memory marker (45) and CCR7, a T cell trafficking marker (8), by multicolor flow cytometry (**Figure 8A**). Though not significant, central memory cells in both CD4 and CD8 compartments exhibited a decreasing trend in the percentage of cells in patients achieving CR, and effector memory cells showed an increasing trend in patients achieving CR compared to those not achieving CR (**Figure 8B**). We only observed significantly lower activated T cells at diagnosis in patients achieving CR following RVD treatment (14.1±2.6) ($p<0.05$) compared to patients not achieving CR (35.6±3.7) (**Figure 8B**). Relatively, absolute cell counts showed significantly higher CD4 and CD8 naïve memory cells in patients achieving CR compared to those not achieving CR. (**Figure 8C**). However, absolute numbers did not display significance for the activated CD3 cell population. (**Figure 8C**).

Determining T Cell functionality using cytokine profiles at diagnosis in patients achieving CR compared with patients not achieving CR following RVD treatment in MM.

The gating strategy on T cells involved analysis of CD4 and CD8 populations, using IFN-γ and IL-17, with PBMCs obtained at diagnosis following six-hour stimulation. This aided in distinguishing Th1, Th17, Th2, and Th1/17 cells within each population (CD4/CD8) (**Figure 9A**). Within these cell populations, significance was observed in Th1 cells, where the percent of CD4 cells was lower at diagnosis in myeloma patients achieving CR (23.3±5.6) ($p<0.05$) compared with patients not achieving CR (46.3±5.4). In addition, Th2 levels in the CD4 compartment were also significantly higher in patients achieving CR compared to those not achieving CR (73±6.1 vs. 47.7±5.9, respectively, $p<0.05$) (**Figure 9B**). Though similar trends were observed in the CD8 compartment, they were not statistically significant. Incorporating

absolute cell counts, a similar change in Th2 cells in the CD4 compartment was observed in patients achieving CR compared to those not achieving CR (**Figure 9C**). Conversely, Th1 cells were not significantly different between these two groups of patients when considering absolute cell counts. (**Figure 9C**).

Frequency of regulatory T cells at diagnosis of MM patients achieving CR compared with patients not achieving CR observed after RVD treatment.

Quantification of regulatory T cells was performed using PBMCs obtained at diagnosis, following RVD treatment. Regulatory T cells were classified as $CD4^{+}/CD127^{low}/CD25^{high}/FOXP3^{+}$ cells by the gating scheme shown in **Figure 10A**. CD127 and IL-25 within CD4 population were used to recognize the Treg cell population, after which further specification was used by staining for Foxp3, a master transcription factor for Tregs (46), and CD4 specific gating with Foxp3 gave the cells the identity of $CD4^{+}Foxp3^{+}$ cells (**Figure 10A**). After selective gating, a trend, though insignificant, was observed between patients achieving CR compared to those not achieving CR (non-CR) (**Figure 10B**). Furthermore, $CD4^{+}Foxp3^{+}$ quantification showed lower levels of Tregs, but again, there was no statistical significance (**Figure 10B**). Within absolute cell number analysis, we did not observe significant differences in $CD4^{+}/CD127^{low}/CD25^{high}$ Tregs, as well as in $CD4^{+}Foxp3^{+}$ cells, though they are important in immune homeostasis (46) (**Figure 10C**).

T cell Activation markers at diagnosis in patients achieving CR compared with patients not achieving CR following RVD treatment.

T cell activation markers were analyzed by flow cytometry, and the percent of CD4 or CD8 T cells was quantified for each activation marker. In the CD4 compartment, 4-1BB was significantly lower in patients who achieved CR compared with patients not achieving CR following RVD treatment (22.7±7.9 vs. 54.2± 6.2, respectively; $p<0.05$) (**Figure 11A**). In the CD8 compartment, PD-1 was significant, where patients who achieved CR had higher levels compared to patients who did not achieve CR (63.2±8 vs. 39.5±6.1, respectively; $p<0.05$). (**Figure 11A**). Absolute numbers showed significance in both CD4 and CD8 PD-1 compartments, where both were higher in patients that achieved CR. However, 4-1BB was not significant in the analysis of absolute cell numbers (**Figure 11B**).

2.4 Figures

Figure 5. Profiling of B cell developmental stages ascertains differences between MM BM versus Normal BM (NBM).

(A) Flow cytometry analysis of the early B cell populations in normal bone marrow (NBM) compared to smoldering myeloma (SMM), myeloma (MM), and relapse/refractory myeloma (RRMM). Multicolor antibodies were used as listed in **Table 1**: Precursor B cells. Gates are set on cells that are classified by *Hystad et. al.* (21), as follows: observed populations are pro-B ($CD34^+$ $CD10^+$ $CD19^+$ IgM^-), pre-B ($CD34^-$ $CD10^+$ $CD19^+$ IgM^-), and Immature B cells (IM-B) ($CD34^-$ $CD10^+$ $CD19^+$ IgM^+). Percentages in each quadrant represent the percent of each cell type, out of the percent of cells in this representative dot plot analysis. **(B)** Number (N) of patient samples obtained in each group (written next to the type of MM). Percent of $CD34^+/CD38^+/CD10^+/CD19^+$ cells in each stage of MM from the flow diagram in (A) are represented as a bar graph. Error bars represent the mean and standard error of the mean (*$p<0.05$).

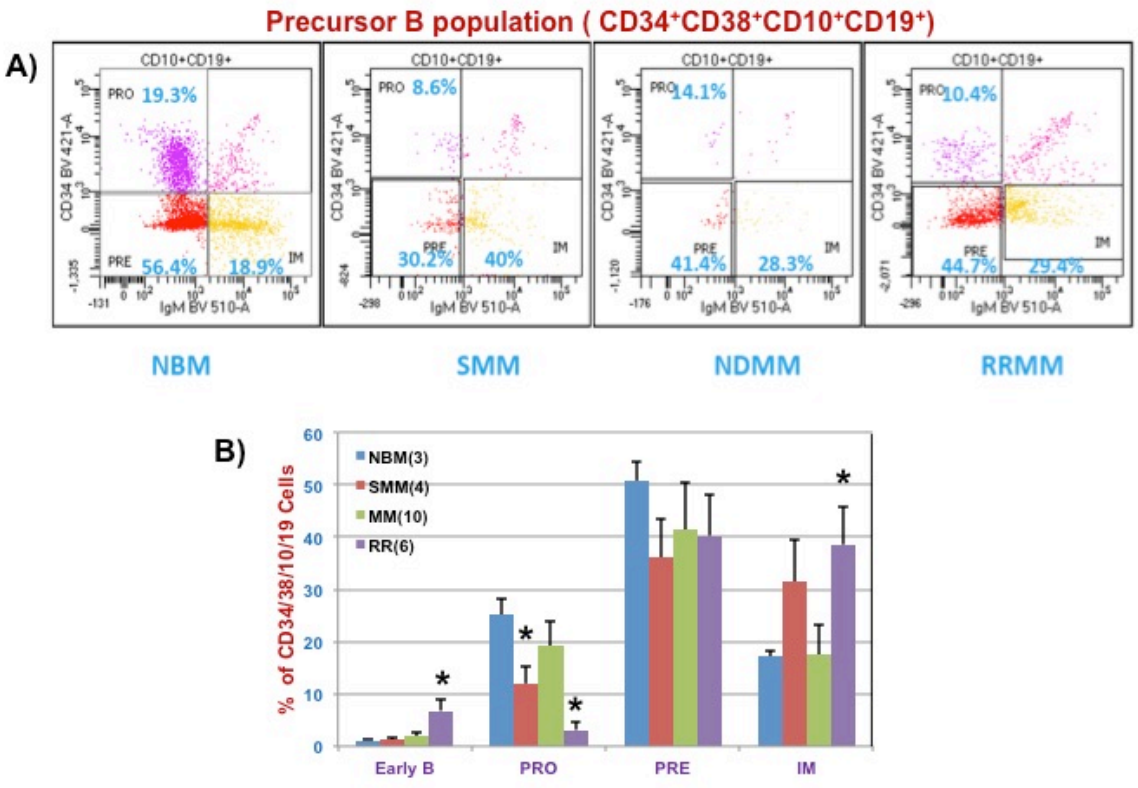


Figure 6. B cell Subset quantification at diagnosis in patients achieving CR following RVD treatment compared with patients not achieving CR.

(A) This representative panel shows flow cytometry analysis of four B cell subsets based on the distribution of markers for CD43, and CD5 in $CD19^+$ B cells. Multicolor antibodies were used as listed in **Table 1**: B cells. Isolated populations include B1a ($CD19^+CD43^+CD5^+$), B1b ($CD19^+CD43^+CD5^-$), B2 ($CD19^+CD43^-CD5^-$), and Bregs ($CD19^+CD43^-CD5^+$). **(B)** Quantification of $CD19^+$ B cells in 6 B cell subsets in addition to memory and follicular B cells (FB) in patients achieving CR (N=13) versus those not achieving CR (N=18) from the 10-106 study. **(C)** Quantification of absolute numbers of B cell-subsets per ml in subset of patients from (B). Error bars represent the mean and standard error of the mean (*p<0.05).

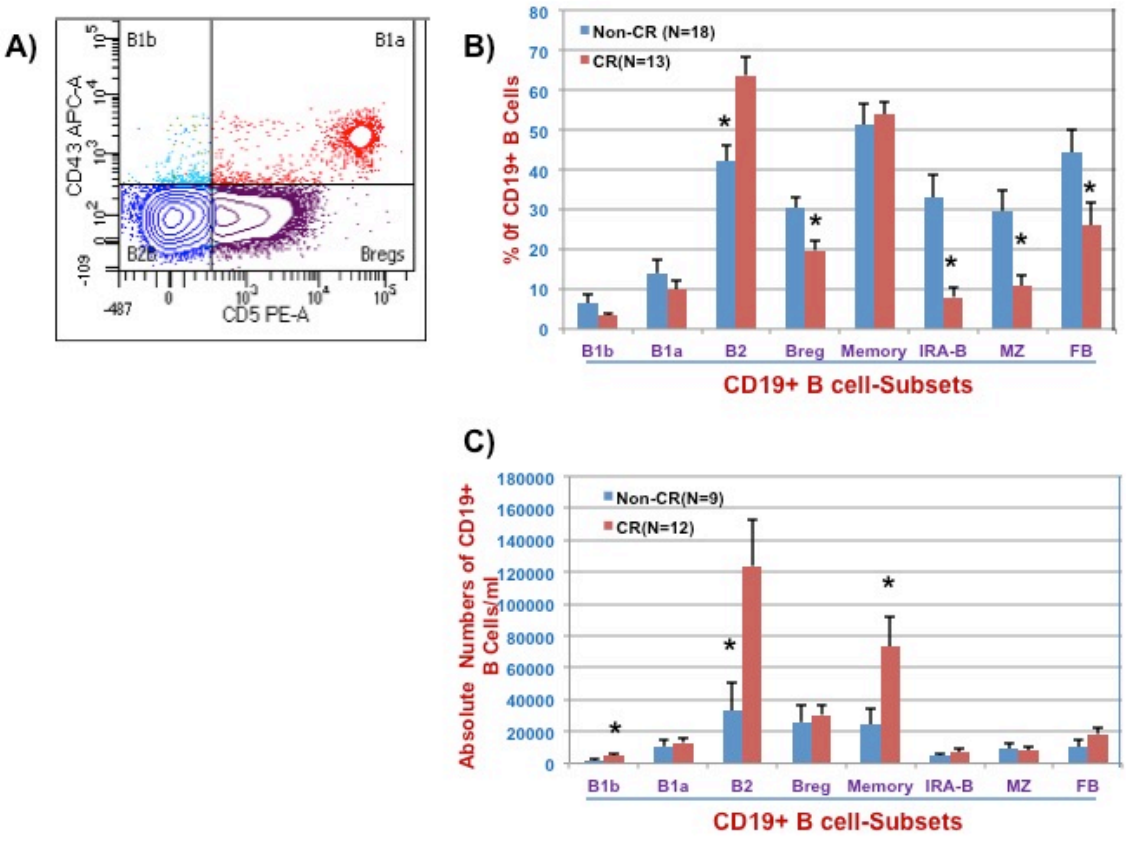


Figure 7. Comparative frequencies of T, B, NK and CD4 cells at diagnosis in patients achieving CR compared with patients not achieving CR.

(A) Gating strategy for flow cytometry analysis, to identify distinct cell populations: CD4 ($CD4^+CD8^-$), CD8 ($CD4^-CD8^+$), and NKT ($CD4^-CD8^-$) within $CD3^+$ lineage cells. Multicolor antibodies were used as listed in **Table 1**. **(B)** Average % of lymphocytes, T cells ($CD3^+$), CD4 cells, CD8 cells, NKT cells, as well as B cells and NK cells in PBMCs, at diagnosis in MM patients achieving CR (N=13) and not achieving CR (N=18) following RVD treatment. **(C)** Absolute cell numbers per ml for each cell type in subset of patients from (B). Error bars represent the mean and standard error of the mean (*p<0.05).

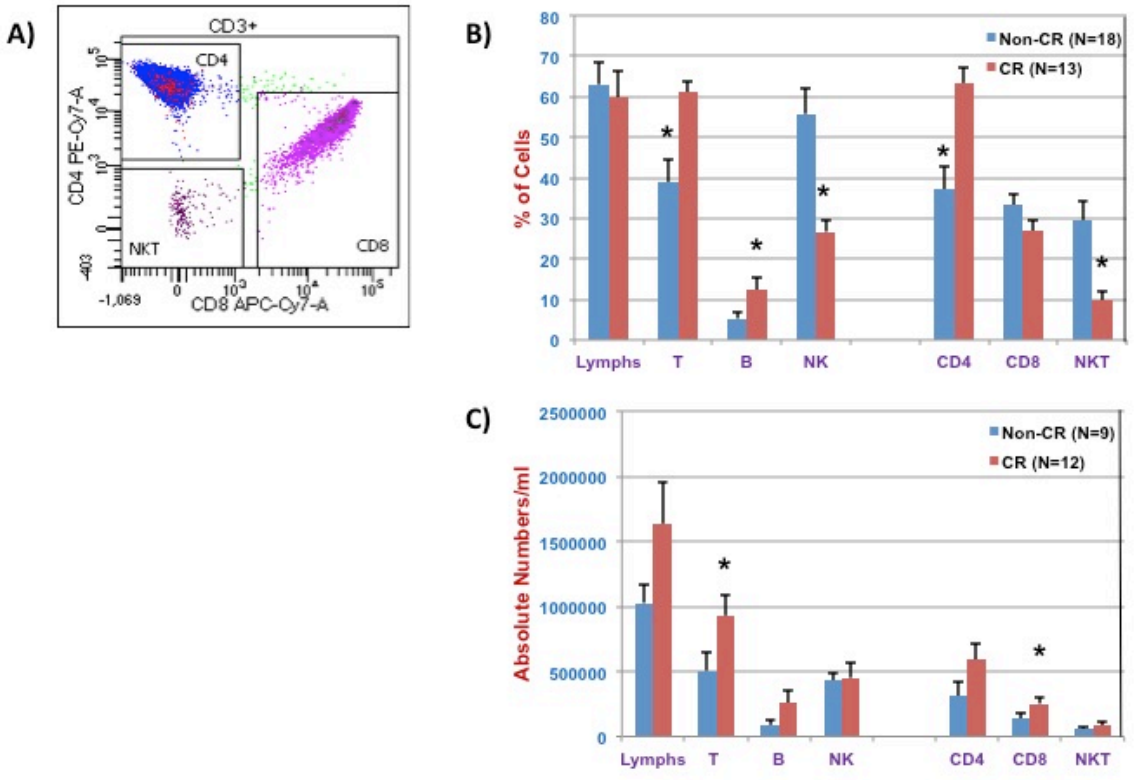


Figure 8. Quantification of circulating memory T cell-subsets in CD4 and CD8 populations at diagnosis in patients achieving CR compared with patients not achieving CR following RVD treatment in MM.

(A) Representation of the gates set up for flow analysis of memory cell populations using CCR7 and CD45RA in both CD4 and CD8 cells. Central memory (CM) cells ($CCR7^{+}CD45RA^{-}$), Effector memory (EM) cells ($CCR7^{-}CD45RA^{-}$), Naïve memory cells ($CCR7^{+}CD45RA^{+}$), and Exhausted memory cells ($CCR7^{-}CD45RA^{+}$). Multicolor antibodies were used as listed in **Table 1**: T cells. **(B)** Shown is quantification of the percentages of cells in each of the four memory compartments, within CD4 and CD8 cells, as well as overall activation of $CD3^{+}$ cells at diagnosis in patients achieving CR (N=13) versus patients not achieving CR (N=18) following RVD treatment. **(C)** Absolute cell numbers per ml for each cell type were identified for the subset of populations mentioned in (B). Error bars represent the mean and standard error of the mean (*$p<0.05$).

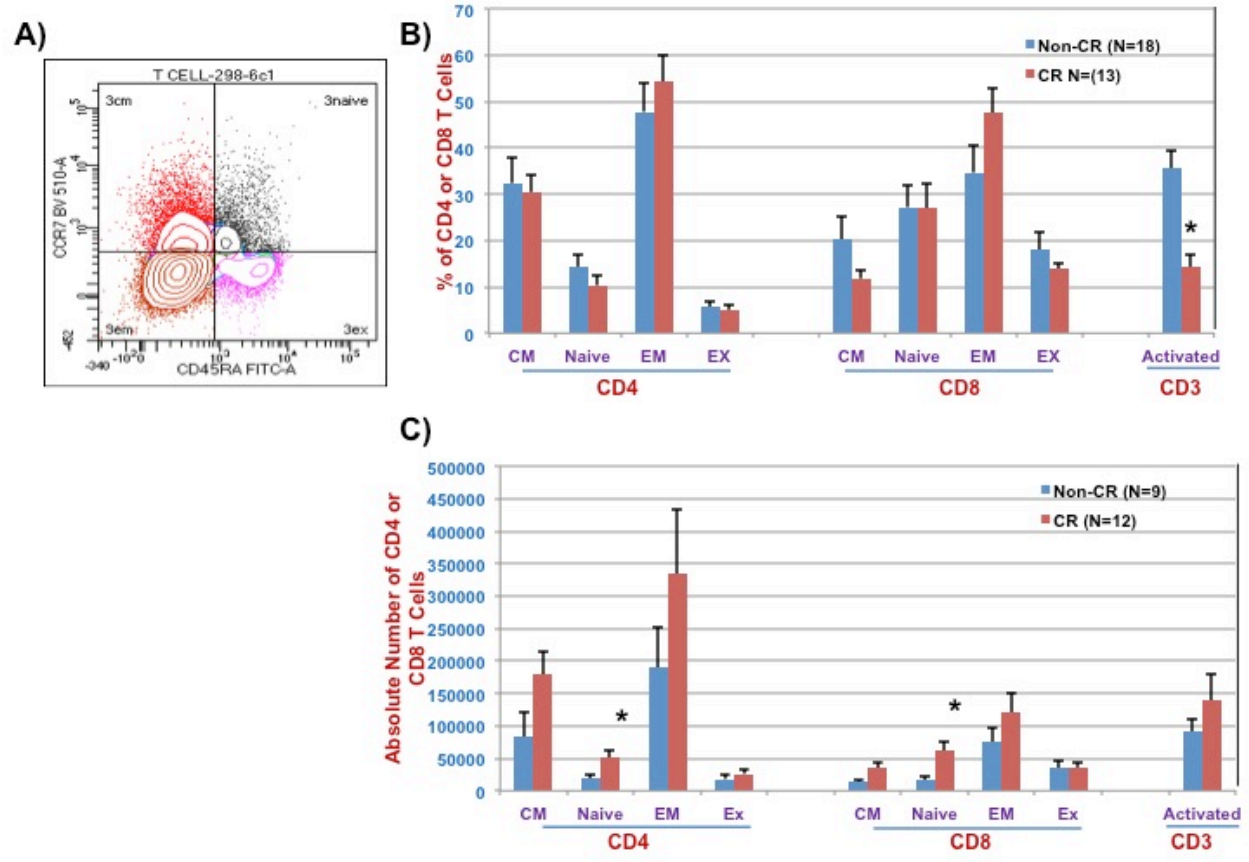


Figure 9. Determining T Cell functionality using cytokine profiles at diagnosis in patients achieving CR compared with patients not achieving CR following RVD treatment in MM.

(A) Flow plots showing the gating strategy within CD4 and CD8 cells, using cytokine signatures IFN-γ and IL-17 to distinguish Th1 cells ($IFN\gamma^{+}IL17^{-}$), Th2 cells ($IFN\gamma^{-}IL17^{-}$), Th17 cells $IFN\gamma^{-}IL17^{+}$), and either Th1/Th17 cells ($IFN\gamma^{+}IL17^{+}$) (43). Multicolor antibodies were used as listed in **Table 1**: Cytokines. **(B)** Quantification of four subpopulations from (A) in patients achieving CR (N=13) versus not achieving CR (N=18) following RVD treatment. **(C)** Absolute cell numbers per ml for each cell type for a subset of patients mentioned in (B). Error bars represent the mean and standard error of the mean (*p<0.05).

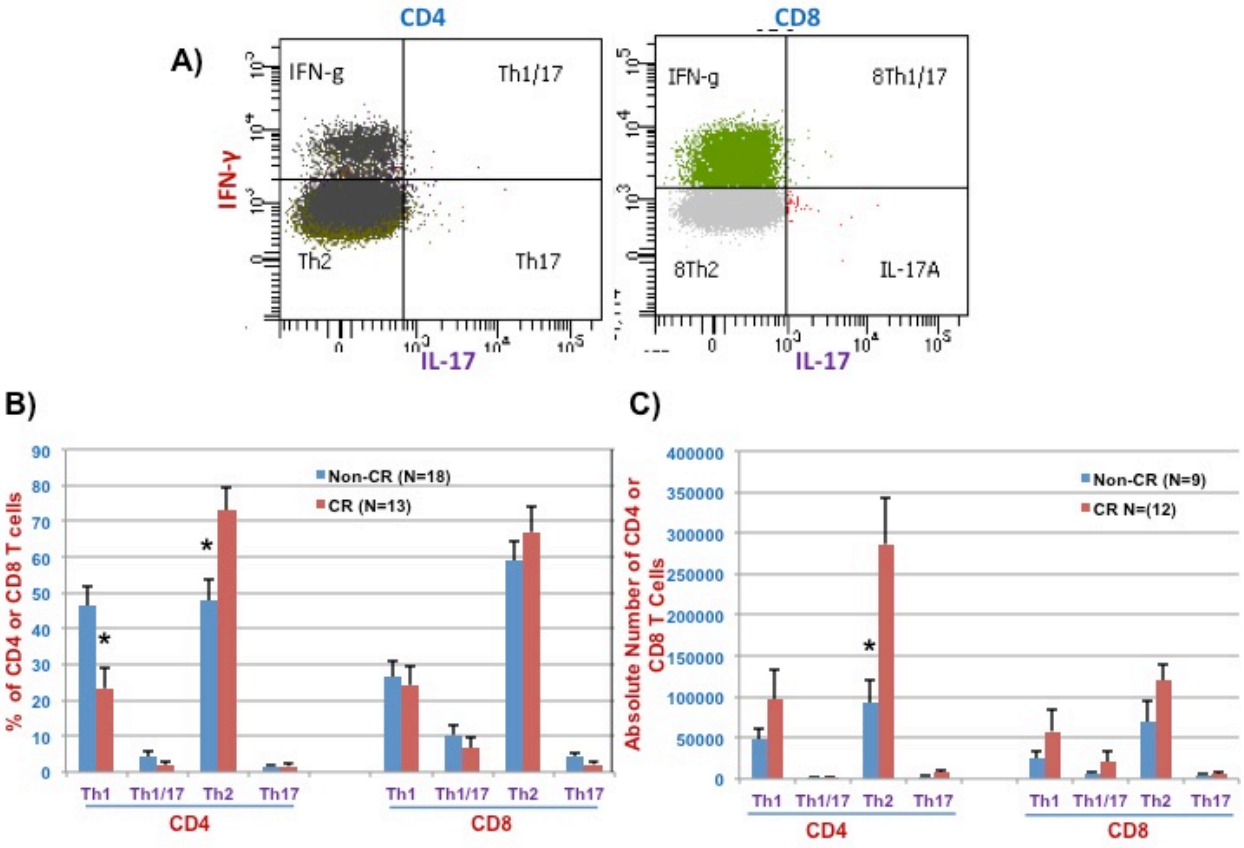


Figure 10. Frequency of regulatory T cells at diagnosis of MM patients achieving CR compared with patients not achieving CR observed after RVD treatment.

(A) Gating strategy for flow cytometry analysis, using CD127 and CD25 markers for identification of the Treg population ($CD127^{-}CD25^{+}$) (42). Then the Treg population was gated on with Foxp3 and CD4, to further identify Tregs. Classification of $CD4^{+}Foxp3^{+}$ Tregs was done by gating on CD4 within the CD3 population from the middle plot in (A), and Foxp3. Multicolor antibodies were used as listed in **Table 1**: Tregs. **(B)** Shown is the quantification of the % of Treg population in patients achieving CR (N=13) versus patients not achieving CR (N=18). In addition, the smaller graph shows $CD4^{+}Foxp3^{+}$ cells in addition to $FOXP3^{+}CD8^{+}$ and $FOXP3^{+}NKT^{+}$ from $CD3^{+}$ population in patients who achieved CR vs. those who did not achieve CR from (A). **(C)** Absolute cell numbers per ml for each cell type for a subset of patients mentioned in (B). Error bars represent the mean and standard error of the mean (*$p<0.05$).

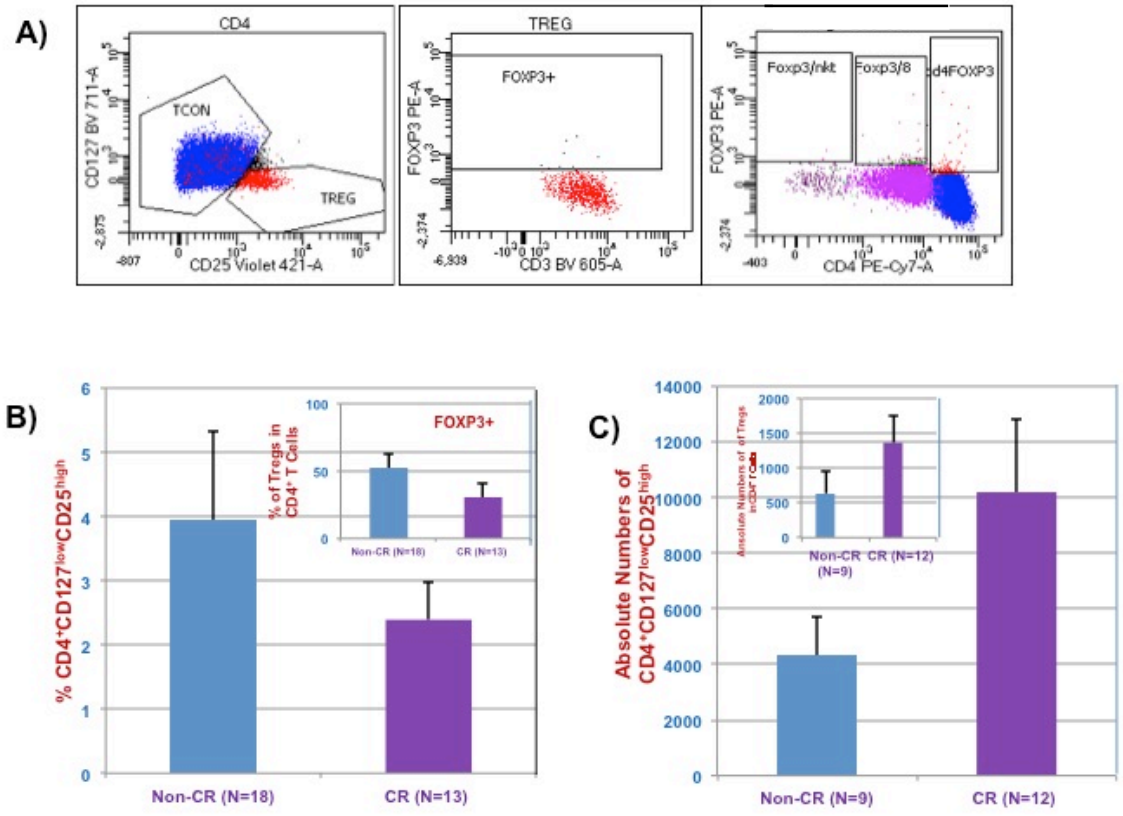
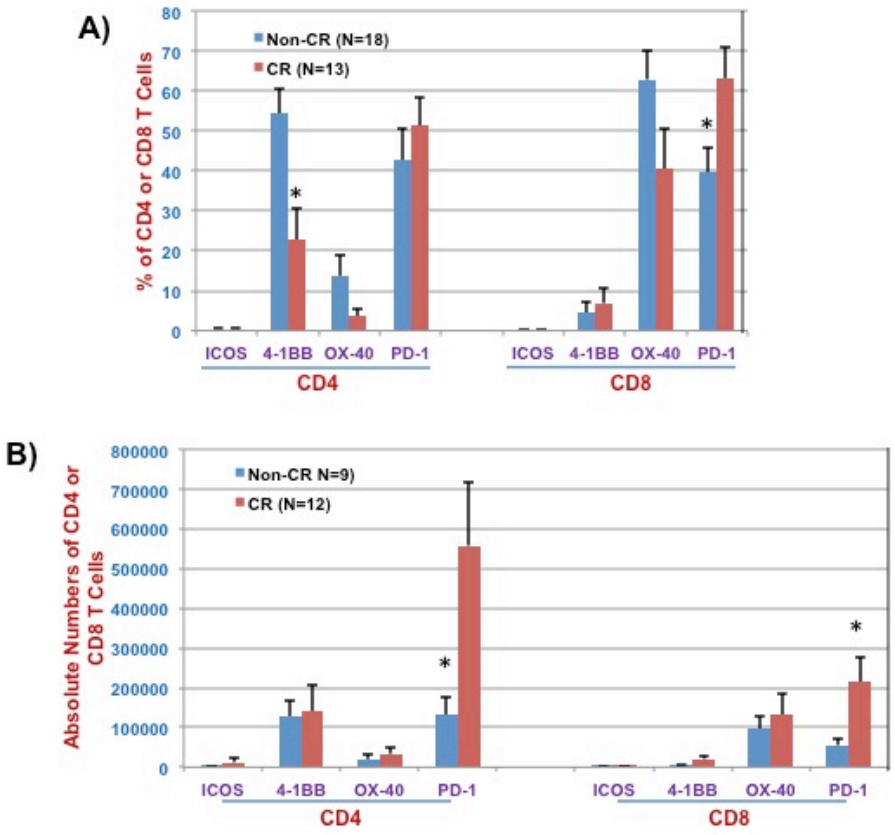


Figure 11. T cell Activation markers at diagnosis in patients achieving CR compared with patients not achieving CR, following RVD treatment in MM.

(A) Percentage cells expressing activation markers: ICOS (CD278), 4-1BB (CD137), OX-40 (CD134), and PD-1 (CD279) in Both CD4 and CD8 compartment in patients achieving CR (N=13) versus not achieving CR (N=18) following RVD treatment. Multicolor antibodies were used as listed in **Table 1:** Activation. **(B)** Absolute cell numbers per ml for each cell type for a subset of patients mentioned in (B). Error bars represent the mean and standard error of the mean (*p<0.05).



2.5 Table(s)

Table 1

List of Conjugated Monoclonal Antibodies (BD Biosciences) used to stain Peripheral Blood Mononuclear Cells and Bone Marrow Mononuclear Cells for Flow Cytometry Analysis with six different tubes.

These antibodies were used to stain frozen PBMC's and BMMC's in five separate tubes, including, B cells, T cells, Tregs, cytokines, and activation; and last tube for precursor B cells using freshly collected bone-marrow samples as stated in the Materials and Methods.

Conjugated Monoclonal Antibodies (BD Biosciences)					
B Cells	**T Cells**	**Tregs**	**Cytokines**	**Activation**	**Precursor B Cells**
CD19	CD3	CD3	CD3	CD3	CD34
CD5	CD4	CD4	CD4	CD4	CD38
CD43	CD8	CD8	CD8	CD8	CD10
CD23	CD45RA	CD127	IFN-γ	CD134 (OX-40)	CD19
CD27	CD197 (CCR7)	CD25	IL-4	CD137 (4-1BB)	IgM
CD185 (CXCR5)	CD69	FOXP3	IL-9	CD154 CD40)	CD22
GM-CSF			IL-17A	CD278 (ICOS)	CD43
				CD279 (PD-1)	CD365 (Tim-1)
					CD24
					IgD

Chapter Three: Discussion and Perspectives

3.1 Discussion

Existing treatments for multiple myeloma, such as chemotherapy and bone marrow transplant, are extremely effective but not curative. The revolution of immunotherapy with immunomodulatory drugs, in combination with a number of targeted therapies (including checkpoint blockade), has paved the way for more fruitful, convenient therapeutic strategies for myeloma patients.

How immune cell profiles in the tumor microenvironment after treatment protocols can be used to predict response has seldom been studied. However, a few studies display that assessing tumor infiltrating immune profiles before and after treatment, can in fact be manipulated to predict clinical response (18). Pursuing such seemingly original insights, our endeavor to uncover a piece of the puzzle that is surprisingly not well understood in the context of myeloma, deficient humoral immunity, proves successful. Outlining significant variations in multiple immune cell subsets paves the way not only for observations of immune cell responses, but also quantification of plausible predictive capabilities for therapeutics, for application directly at diagnosis, for MM patients. Our data acknowledges trends in B cell developmental compartments in MM bone marrow, as well as in B and T cells in the peripheral blood of myeloma patients. Specifically, limited knowledge of humoral immunity, UIgs, and immune cell responses in multiple myeloma contributes greatly to the novelty of our approach.

To begin to delve into the causative factors behind low observed uninvolved immunoglobulins, our observed defects in B cell lineage developmental compartments aid in understanding the role of invasive myeloma cells in the tumor microenvironment, causing

hindrance to effective humoral immunity (i.e. low B cell production in MM). As myeloma progresses, the differences between normal bone marrow and myeloma bone marrow, in terms of B cell development, are major (**Figure 5A**). Since pro-B and pre-B cells in myeloma bone marrow are lower than normal, it can be inferred that tumor cells are interfering with B cell development prior to germinal center maturation. Primarily, differences in B cell developmental profiles between normal bone marrow and myeloma patient bone marrow arise as low levels of pro-B cells, compared to normal bone-marrow cells (**Figure 5B**). In correlation, depleted mature B cell counts in myeloma (17, 24) may be due to the hindrance of such progenitor-like, stem cell, renewal capabilities that pro-B cells possess. Additionally, although it beckons further clarification, notably low percentages of B2 cells at diagnosis in MM patients achieving CR compared to patients not achieving CR after RVD treatment (10-106 study) (**Figure 6B, 6C**), who are more likely to relapse, associate with patterns of low pro and pre B cells in development (**Figure 5**). Hence, since the B2 subset is responsible for encompassing the majority of conventional B cells, interference in development may be triggering skewed developmental profiles in the MM bone marrow. Accordingly, the suppression of UIgs may most likely be affected due to this interference in the bone marrow microenvironment, where abnormal plasma cells are stationed. We also observed an increased population of immature B cells in the bone-marrow samples collected from RRMM patients, which may indicate that tumor cells in the myeloma bone marrow microenvironment accelerate the B cell developmental process, thereby generating a higher number of immature B cells (**Figure 5**).

Uncovering differences by simply observing significant immune parameters at diagnosis, in myeloma patients who achieved CR compared to those who did not achieve CR, gives scope to establish significant immune signatures, which can aid in prospective therapeutic predictions

of CR when implementing a RVD treatment strategy. Intriguingly, of all the immune subsets that were examined, quantification of B2 cells in B cell subsets, T cells in analysis of lineage markers, Th2 cells through cytokine analysis, and CD8 specific PD-1 activation are consistently significant in both compositional cell-percentages, as well as absolute cell count analyses (**Figures 5-11**). Higher B2, Th2, T, and CD8 PD-1 profiles at diagnosis, in patients who achieved CR after receiving RVD treatment indicates role of normal immune function at diagnosis in influencing clinical outcome after therapy. Implications of defining such profiles are, for instance, that notably high Th2 levels at diagnosis itself in patients who achieved CR after RVD treatment suggest more stability (**Figure 9**), since a Th1/Th2 imbalance has been identified in myeloma (32, 33). Hence, MM patients who achieved CR following RVD treatment may exhibit more stability, and potentially have longer PFS (progression-free survival) and/or a less chance of relapse compared to patients who achieved non-CR after RVD treatment, as previously defined. Th2 cells are also close cognitive responders to provide B cell help, and therefore have associations in the context of myeloma, as B2 and Th2 cells are at significantly high levels in patients who achieved CR (13, 51). Moreover, it is known that activated CD8 cells normally express PD-1 (49). The high PD-1 levels in the CD8 T cell compartment attests to not only restoration of activation relatively closer to normal and/or at exhausted levels in patients who achieved CR compared to those who did not achieve CR, but also correlates with the significance of CD3 activated cells (**Figure 8, Figure 11**). Though imminent trends were observed, there was no significance seen in Tregs and memory cell populations that were consistent with both aspects of analysis, likely due to caveats discussed in 3.2. Nevertheless, the retrospective experimental significance of these four parameters (B2, Th2, T lineage, CD8 PD-1)

augments their credibility for use as predictive baselines to evaluate prospective CR status in MM patients.

Recent advances in B cell-biology indicate that besides generating antibodies, B cells have much larger role in generating and regulating protective immune responses, particularly cell-mediated (30, 31). Multiple myeloma causes abnormal behavior in B cell subset populations, leading to B cell dysfunction, and the observed abnormalities in humoral immunity. Applying immunomodulatory drugs to these profiles shows promise in restoring the B cell compartments. The ability to identify significantly altered immune cell subsets after RVD treatment, suggests that predictive potentials may be able to efficiently improve therapeutics for myeloma patients following the time of diagnosis itself. Here, we have discerned that MM cells do impact precursory B cells, through undefined mechanistic interactions in the bone marrow, and propose that these differences can begin to provide rationale for low uninvolved immunoglobulin levels seen in MM. Tying together the fundamental questions to effectively classify immune cells in myeloma phenotypes, we have defined immune parameters that have the capacity to influence foreseeable therapeutic response for MM treatments (18).

3.2 Limitations

Establishing immune cell profiles becomes principally important, as identifying behavioral aspects in the MM tumor microenvironment must be well correlated with prediction of clinical response. In the 10-106 study, multiple caveats necessitate further validation. We are currently blinded regarding the type of treatment patients received in the US study (RVD-alone vs. transplant+RVD). Uncovering this may provide greater insight into whether these differences

in therapy can affect immune function and also affect the overall outcome, which was observed in the recently published IFM part of the study. This information will not be revealed until the study is completed. However, we have the response data (CR versus less than CR) for correlation with immune status at diagnosis.

Furthermore, in the overall study incorporating developmental profiles, as well as B and T cell signatures, the small sample sizes can account for the insignificant, yet plausible trends seen in cell populations. For instance, absolute cell numbers are significantly higher in patients who achieved CR for B1b cells, showing a strong correlation for validity, yet B1b cells become insignificant in quantification of CD19 cell counts, likely due to sample size (**Figure 6B, 6C**). Amongst the two approaches used to examine immune profiles, by traditional cell percentages and absolute cell counts, many differing trends are observed. For instance, CD8 cells in the identification of lineage markers (**Figure 7B**) are lower in patients who achieved CR compared to those who did not achieve CR, yet are not significant. However, absolute cell numbers show significance in CD8 cells, which are higher in patients who achieved CR (**Figure 7C**). Because absolute cell numbers were only available for 9 patients who did not achieve CR and 12 patients who achieved CR, out of 18 and 13, respectively, the data may be partial. The constraint of small sample sizes also limits our confidence in the analysis of B cell developmental compartments. In this case, small sample sizes in MM BM samples are due to the duration of time required to obtain fresh bone marrow samples (**Figure 5**).

Finally, establishing a baseline of immune parameters to predict response in MM is particularly advantageous, though another caveat presents itself in terms of universal applicability of these criteria to another treatment protocol. This study solely defines prognostic parameters for patients who receive RVD treatment with and without transplant, and is not

relevant to other immunomodulatory drugs. Consequently, every time an immunomodulatory and/or combinatorial therapy is given, analyses specific to that treatment will need to be performed to yield predictive prognosis parameters at diagnosis. Although multiple stipulations are established through this study, it is beneficial to pursue these shortcomings to ascertain further advances in this novel idea.

3.3 Future Directions

As basis for these analyses, the long-term, primary goal is to alleviate B cell-abnormalities and improve humoral immunity in myeloma, for the generation of robust anti-myeloma responses. More importantly, improving B cell function will help prevent infectious complications and thereby quality of life (higher PFS), and will also aid in improving T cell function, allowing for the development of T cell responses against myeloma.

Following this study, the seemingly most important step of future work is determining if there are differences between the transplantation+RVD group and the RVD-alone group of patients. Then, comparative studies can follow, regarding the advantages of therapeutic strategies and measurement of progression-free survival after CR prediction**.** In addition, similar trends in signature transcription factors for the immune cell populations analyzed here, can aid in validation of the data presented. Prospective studies will be to evaluate the B and T cell repertoire by deep sequencing to profile rare clonal heterogeneity (12). For instance, profiling patients in the initial MGUS stage to newly diagnosed myeloma, and observing precursory B cell compartments as well as B and T cell interactions (i.e. germinal center), will uncover mechanistic insight into the development of malignancy.

Based on the studies identified in this paper, we hope to increase our sample size to classify even more baseline parameters, if deemed significant, that may yield a predictive potential for the RVD treatment to ascertain CR or not. Further inquiry could lead to establishing protocols for immune cell analysis that are universally applicable to the evolving combinatorial therapeutic strategies in MM. Current treatment protocols in this field can be supplemented by exploring treatment combinations which not only bring patients to CR, but rather, restore robust immune cell compartments that bring relapse rates to a minimum. Eventually, these analyses hope to supplement advanced therapeutic strategies, such as immunomodulatory drugs in combination with checkpoint blockade, to alleviate B cell-abnormalities in multiple myeloma.

Here, even interplay between B and T cell interactions can bring forth significant correlations if patient sample pools in each group (CR vs. non-CR) are expanded. How these altered immune cell-subsets participate in the increase of genomic instability, mutational load, and MM plasma cell clonal heterogeneity during myeloma progression, will answer further questions regarding the composition of these subsets, and provide a guideline for eliminating clonal resistance to therapeutics, and hence relapse of MM.

Having a predictive capacity for current therapeutic interventions, besides providing deep clinical responses, may improve humoral immunity, and hence, impact on long-term patient outcome (PFS). By uncovering behavioral modalities of different immune cell compartments, immune dysfunction can be further classified, so that developing immunotherapy protocols with the established results (i.e. parameters defined in this study) can hold their predictive credibility when targeting multiple age groups in the relapse-prone MM population.

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